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Sustaining N_2-Dependent Growth in the Presence of CO▽§

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Low levels of carbon monoxide inhibit the N_2-dependent growth of *Rhodospirillum rubrum* unless the ~100-residue CowN protein is expressed. Expression requires the CO-responsive regulator RcoM and is maximal in cells grown in the presence of CO and a poor nitrogen source, consistent with the role of CowN in N_2 fixation.

The uniquely prokaryotic process of nitrogen fixation depends upon a highly regulated and elaborate enzyme complex to effect the energy-intensive reduction of N_2 to NH_3. Aside from the *nifHDK* structural genes that encode the dinitrogenase catalytic complex, additional factors execute the assembly of its unique metal centers and enable protein maturation and stabilization, while other *nif*-coregulated gene products remain functionally undefined (6, 19, 20). As this elaborate synthesis and function are inhibited by the prevalent environmental gases O_2 and CO (4, 7), it is reasonable that some accessory factors might prevent enzyme damage or effect repairs should it occur. Indeed, the "Shethna" protein has been shown to associate with dinitrogenase, forming an inactive but O_2-tolerant complex (5). No factors are known to specifically protect against inactivation by CO.

We noted that some genomes bear a conserved open reading frame (cORF) immediately downstream of genes encoding RcoM, a newly discovered CO-responsive regulator (8). Here we report detection of this cORF in a configuration similar to that of genes for another CO-responsive regulator (CooA) or within *nif* operons, and we show that in *Rhodospirillum rubrum* the encoded protein, hereby designated CowN (CO Weal-Nitrogenase), protects this organism's ability to fix N_2 in the presence of CO.

Database searches employing the PSI-Search function (SSEARCH ver. 35.04 [http://www.ebi.ac.uk/] [16, 22]) with CowN from *R. rubrum* as a probe indicated 33 similar proteins, averaging 98 residues in length (current as of 30 March 2010; see Table SA1 in the supplemental material). A representative alignment of CowN proteins from diverse organisms is presented in Fig. 1, and the complete alignment is shown in a supplement (see Fig. SA1 in the supplemental material). All organisms bearing *cowN* also contained *nifHDK* genes and are known or presumed to be capable of N_2 fixation. In nine instances *cowN* was preceded by *cooA* and a probable CooA binding site (tGTCg-[6n]-tGACa; lowercase letters designate less-conserved residues), in six instances it was preceded by *rcoM* and a probable RcoM-binding sequence (a triple direct repeat of cngTTnnnG-[11-12n]), and in four instances *cowN* appeared to be transcriptionally linked to possible *nif* genes. In 14 other instances *cowN* is genomically isolated, unlinked to *nif* genes or nearby genes encoding DNA-binding regulators; in fact, most of these organisms appear to lack the genes for CooA and RcoM (Table SA1). We hypothesized that N_2 fixation in the presence of CO would depend upon *cowN* expression because of (i) the genomic concurrence of *cowN* and *nif* genes, (ii) the indication of occasional transcriptional linkage of *nif* and *cowN* genes, (iii) the frequent juxtaposition of genes encoding the CO-responsive regulators RcoM or CooA with *cowN*, and (iv) the known inhibition of nitrogenase by CO.

To test this hypothesis, we evaluated the growth of *R. rubrum* in a defined medium containing (per liter of purified water): 8.4 g morpholinopropanesulfonic acid (adjusted to pH 6.8), 0.1 mg biotin, 0.2 mg *p*-aminobenzoic acid, 250 mg $MgSO_4 \cdot 7H_2O$, 100 mg $CaCl_2 \cdot 2H_2O$, 200 μM EDTA, 50 μM H_3BO_3, 10 μM $FeSO_4 \cdot 7H_2O$, 2 μM $Na_2MoO_4 \cdot 2H_2O$, 1 μM $CoCl_2 \cdot 6H_2O$, and 2 μM $MnCl_2 \cdot 4H_2O$. Note that this formulation omits nickel to diminish CO utilization by the Ni-dependent *coo* system (10). Sterile, pH-adjusted (7.0) additions of potassium phosphate (to 10 mM) and DL-sodium malate (to 25 mM) were added to the autoclaved medium, along with a source of nitrogen: addition of NH_4Cl (to 15 mM) or N_2 present in the GasPak jar (O_2 was removed using GasPak Plus envelopes; Becton Dickinson and Company). Plate media were solidified with 1.3% agar, and the growth at ~28°C of four *R. rubrum* strains was compared: the "wild-type" (WT) strain UR2 (10), strain UR616 (Δ*cooA*::*aacC1*Ω), and newly created strains bearing the chromosomal alterations *rcoM*::*aacC1*Ω*linker* (designated UR2216) and *cowN*::*aacC1*Ω (UR2546). These strains were constructed by typical methods (10) and verified by amplification of the altered chromosomal region, agarose gel analyses, and sequencing of the PCR products (data not shown).

In the presence of ammonium all strains grew equivalently in aerobic and anaerobic (photoheterotrophic [21]) growth modes, and the addition of CO was without effect (data not shown and Fig. 2A). Strains also grew comparably in photoheterotrophic conditions in the absence of CO when N_2 served as the sole nitrogen source (Fig. 2B) but unequally if a low level of CO (0.15% initial level) was present (Fig. 2C): growth of the WT (UR2) and *cooA* (UR616) strains was superior to growth of the strains in which *rcoM* (UR2216) or *cowN*

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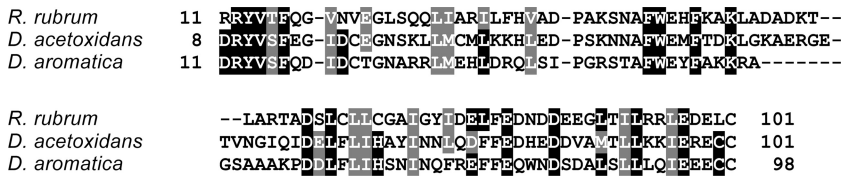

FIG. 1. Conservation of CowN residues (representative subset). This alignment of CowN proteins from alphaproteobacterial, betaproteobacterial, and deltaproteobacterial representatives (*R. rubrum*, *Desulfuromonas acetoxidans* DSM 684, and *Dechloromonas aromatica* RCB, respectively) is based upon a full alignment of 33 proteins (see Fig. SA1 in the supplemental material) generated by the ClustalW2 facility (Clustal version 2.0.12 [http://www.ebi.ac.uk/Tools/clustalw2/] [13]) using the Gonnet 250 matrix. Shading (Boxshade 3.21 [http://www.ch.embnet.org/software/BOX_form.html]) is based upon the full alignment with a 0.7 consensus.

(UR2546) had been insertionally inactivated. In contrast, the WT, *rcoM*, and *cowN* strains grew similarly when cultivated under non-nitrogen-fixation conditions suitable for anaerobic, CO-dependent catabolism (9), unlike the *cooA* strain (UR616), which failed to grow (Fig. 2D). These results were consistent with a model wherein the *cowN* and *rcoM* gene products are not necessary for CO-dependent growth but are involved in protecting N_2 utilization in the presence of low levels of CO.

This protection was confirmed in liquid cultures of *R. rubrum* growing on N_2. Using the medium formulation specified above (20 ml per 120-ml vial), supplemented with phosphate and malate and supplied with an O_2-free N_2 headspace, photosynthetic cultures exhibited N_2-dependent growth with a doubling time of 8.5 h. Addition of CO to 1% to actively growing cultures of WT strain UR2 induced an ~5-hour growth lag, followed by resumption of normal growth, while the same treatment of UR2546 (*cowN*::*aacC1*Ω) inhibited growth for the duration of the experiment (Fig. 3). The growth inhibition appears to be bacteriostatic: viable counts from samples obtained prior to CO addition and at the 12-hour timepoint (Fig. 3) indicated consistent CFU numbers, averaging 3.9×10^8 and 4.0×10^8 CFU/ml/optical density at 680 nm (OD_{680}), regardless of strain or growth condition (99% confidence intervals amounted to less than 10% of these numbers). Samples obtained at 24 h showed similar numbers in actively growing cultures (averaging 4.2×10^8 CFU/ml/OD_{680}) but a 33% decrease in inhibited cultures, either UR2 maintained under argon or UR2546 cultivated with CO (2.9×10^8 and 2.8×10^8 CFU/ml/OD_{680}, respectively). Thus, this decreased viability is not due to a toxic effect of CO. Other results confirm that CO is nontoxic to photoheterotrophic cultures of *R. rubrum*, regardless of the expression of the *coo* system, as seen in Fig. 2A and reported previously (11). The bacteriostatic effect of CO and the kinetics of the WT CO response suggest that CowN must be synthesized and then protect nitrogenase from CO. An alternative hypothesis—CowN-dependent CO removal—would not explain the differential growth of strains seen on plates (Fig. 2C).

Additional *R. rubrum* strains were created to evaluate the requirement of RcoM and CO for *cowN* expression in cells supplied with good or poor nitrogen sources (NH_4Cl or glutamate, respectively; NH_4Cl prevents *nif* expression, while glutamate is an adequate nitrogen source yet allows *nif* derepression). We inserted a promoterless *lacZ*/Km^r cassette (12) to create two *cowN*::*lacZ* transcriptional fusion strains (Fig. 4): one with the WT *rcoM* (strain designated UR2504) and a version with a +1-base frameshift in *rcoM* that created TGA-TAA stops at codons 6 and 7 (UR2630). These constructs were verified by PCR amplification of the altered chromosomal region and sequencing of the products. Liquid cultures were grown photoheterotrophically with mixing at 30°C in the medium specified above but under an argon headspace and with additions of filter-sterilized and pH-adjusted NH_4Cl (to 10 mM) or glutamate (to 25 mM) as nitrogen sources. CO additions to the headspace (4% initial level) were added to cultures at an OD_{660} of ~0.1, and log-phase cell samples were obtained at an OD_{660} of ~1.0 for analysis of β-galactosidase according to standard methods (17). The higher CO level used in this experiment relative to the previous experiments reflects the different (non-N_2-dependent) growth condition and was intended to preclude the possibility that CO utilization could

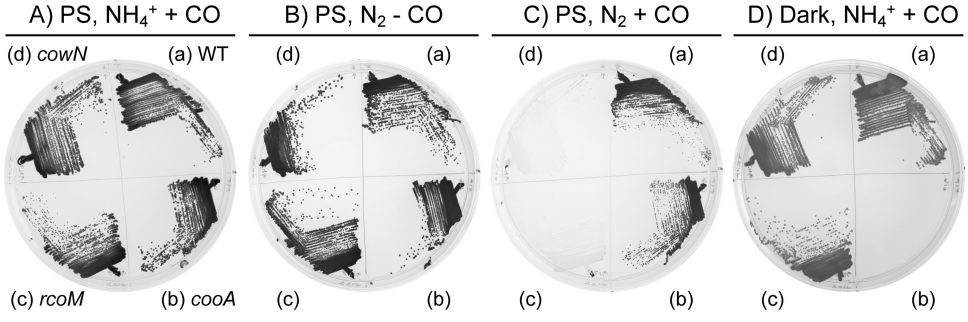

FIG. 2. CowN is required for N_2 fixation in the presence of CO but not for CO-dependent growth. *R. rubrum* strains UR2 (WT), UR616 (Δ*cooA*::*aacC1*Ω), UR2216 (*rcoM*::*aacC1*Ω*linker*), and UR2546 (*cowN*::*aacC1*Ω) were cultivated photosynthetically (PS, plates A to C) on medium supplemented with NH_4Cl in the presence of 0.15% CO (initial level) (A) and on medium requiring N_2 fixation without (B) or with (C) 0.15% CO. Plate D was incubated in the dark under conditions suitable for CO-dependent growth (9).

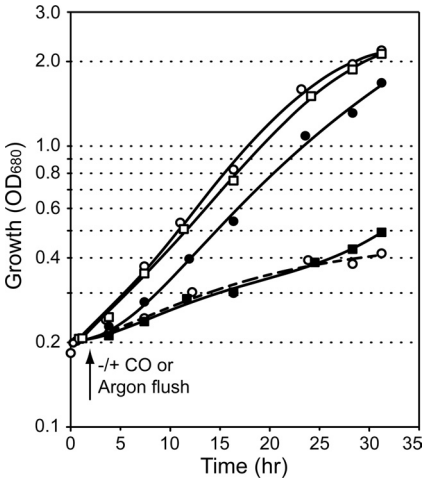

FIG. 3. Expression of *cowN* sustains N_2-dependent growth in the presence of CO. Liquid N_2-dependent cultures of *R. rubrum* strains UR2 (WT, ○ and ●) and UR2546 (*cowN*::*aacC1*Ω, □ and ■), inoculated from N_2-dependent cultures to an OD_{680} of 0.06, were grown to an OD_{680} of approximately 0.2, here designated the zero-hour time point. At approximately 2 h (↑), selected cultures received additions of CO to 1% (● and ■) or the vial headspace was exchanged with argon (UR2 only, dashed line). Each plot represents the average of results for three cultures. Samples for the determination of CFU were obtained at approximately 1, 12, and 24 h (see text).

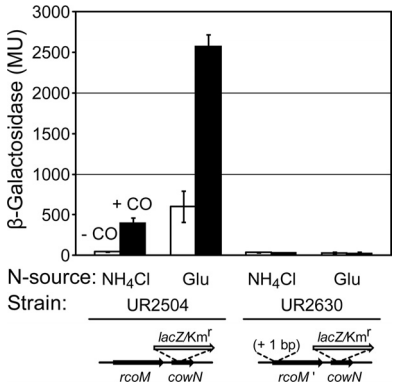

FIG. 4. Expression of *cowN* is CO and RcoM dependent and is enhanced in the presence of glutamate, a nitrogen source that allows *nif* derepression. *R. rubrum* strains bearing a *cowN*::*lacZ* reporter were cultivated under photosynthetic growth conditions in medium containing NH_4Cl or glutamate (Glu), plus or minus added CO (4% initial level). Strain UR2504 bears a WT *rcoM* gene, while in strain UR2630 *rcoM* expression is inactivated by a single-base frameshift at codon 6 that creates dual translational stops at codons 6 and 7. Multiple mid-log-phase cultures were assayed for β-galactosidase, and activities in Miller units (MU) are presented with error bars indicating the 99% confidence interval.

perturb the level of expression, as confirmed by similar results obtained with additions of 2% or 8% CO (data not shown).

UR2504 cultures (*rcoM*$^+$) grown in the presence of CO showed increased *cowN*::*lacZ* expression relative to cultures grown without CO additions, and the level of expression was greatly enhanced when cells were supplied with glutamate instead of NH_4Cl (Fig. 4). In contrast, only slight *cowN*::*lacZ* expression was observed in the frameshifted *rcoM* strain (UR2630), regardless of the nitrogen source or CO addition, consistent with RcoM-dependent promotion of *cowN* expression (Fig. 4). A strain (UR2599) bearing an in-frame 47-codon deletion of the *rcoM* region that encodes the C-terminal DNA-binding domain likewise failed to induce *cowN* expression, again consistent with RcoM-dependent *cowN* expression rather than a polar (e.g., Rho-dependent) transcription effect (data not shown).

The apparently "CO-independent" expression of *cowN*::*lacZ* in strain UR2504 grown in the presence of glutamate suggests the involvement of an additional regulatory mechanism, as does the effect of the nitrogen source on the magnitude of CO-induced expression (Fig. 4). However, at least the "CO-independent" *cowN*::*lacZ* expression could reflect artifactual endogenous synthesis of CO. Indeed, similar growth conditions (malate as a carbon source, glutamate as a nitrogen source, without added CO) were successfully used to cultivate *R. rubrum* for the isolation of carbon monoxide dehydrogenase, the accumulation of which requires CO (1, 2, 3, 21). The dependence of maximal *cowN* expression on (i) a nitrogen source that permits *nif* derepression, (ii) CO (notwithstanding the elevated "minus-CO" baseline for glutamate-grown cultures), and (iii) the presence of RcoM is in accord with CowN protection of nitrogenase function when CO is present (Fig. 2 and 3). The basis of the nitrogen source effect on CO-dependent *cowN* expression is unclear, as binding sites for known *nif* gene transcriptional regulators are not apparent 5′ of *cowN*.

The genomic linkage of *cooA* with *cowN* in other organisms (see Table SA1 in the supplemental material) is likewise consistent with a CO-dependent regulation of CowN. For example, aside from *R. rubrum*, wherein RcoM is necessary for *cowN* expression and CooA regulates expression of the catabolic CO-oxidation (*coo*) system (18), only the sequenced genome of a second photosynthetic bacterium (*Rhodopseudomonas palustris* BisB18) contains both *cowN* and *coo* systems. This organism does not harbor an *rcoM* gene, and the expression of *coo* and *cowN* genes therefore appears to be controlled by dual CooA proteins encoded adjacent to the *coo* regulon (YP_534336) and *cowN* (YP_533050). Presumably, any differential regulation of the *coo* and *cowN* systems relies on different affinities of the regulators for the CO effector or distinctions in target DNA binding. In addition, the biological roles of *coo* and *cowN* systems may reflect nickel availability, for Ni additions are necessary for maximal *coo*-dependent growth (9, 10) while irrelevant for *cowN*-dependent protection of nitrogenase.

CowN joins a list of several accessory functions known or suspected to promote nitrogenase assembly and function (6, 19, 20), specifically by preserving the activity in the presence of CO, a prevalent environmental contaminant. *In vitro* studies will be required to fully elucidate the mechanism of this activity, though a stoichiometric interaction with nitrogenase, not unlike that of the "Shethna" protein (5), seems plausible. That *cowN* is not evident in all organisms possessing a *nif* system may reflect several scenarios: (i) organisms could possess nitrogenase enzymes with inherently different resistances to CO—indeed, a recent report indicates that the vanadium-containing nitrogenase from *Azotobacter vinelandii* can reduce CO (14); (ii) organisms could experience different degrees of CO

exposure or possess efficient mechanisms for its elimination; or (iii) perhaps a CowN-like function is performed by another uncharacterized accessory protein. Besides demonstrating its novel function, the identification of CowN may promote biological hydrogen production processes inasmuch as these rely on nitrogenase (15) and CO inhibits all nitrogenase-catalyzed substrate reductions except for the conversion of $2H^+$ to H_2. Thus, inactivation of *cowN* could increase the degree of CO-bound nitrogenase and thereby focus its catalytic specificity toward H_2 synthesis.

Nucleotide sequence accession numbers. The *R. rubrum* CowN protein sequence is available in databases (NCBI accession number, YP_428597 [http://www.ncbi.nlm.nih.gov/]; UniProtKB accession number, Q2RNI5 [http://www.uniprot.org/]).

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